JOURNAL OF BACTERIOLOGY, Oct. 1998, p. 5421–5425 Vol. 180, No. 20
0021-9193/98/$04.00+0

Fumarate Regulation of Gene Expression in *Escherichia coli* by the DcuSR (*dcuSR* Genes) Two-Component Regulatory System

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Received 26 May 1998/Accepted 10 August 1998

In *Escherichia coli* the genes encoding the anaerobic fumarate respiratory system are transcriptionally regulated by C_4-dicarboxylates. The regulation is effected by a two-component regulatory system, DcuSR, consisting of a sensory histidine kinase (DcuS) and a response regulator (DcuR). DcuS and DcuR are encoded by the *dcuSR* genes (previously *yjdHG*) at 93.7 min on the calculated *E. coli* map. Inactivation of the *dcuR* and *dcuS* genes caused the loss of C_4-dicarboxylate-stimulated synthesis of fumarate reductase (*frdABCD* genes) and of the anaerobic fumarate-succinate antiporter DcuB (*dcuB* gene). DcuS is predicted to contain a large periplasmic domain as the supposed site for C_4-dicarboxylate sensing. Regulation by DcuR and DcuS responded to the presence of the C_4-dicarboxylates fumarate, succinate, malate, aspartate, tartrate, and maleate. Since maleate is not taken up by the bacteria under these conditions, the carboxylates presumably act from without. Genes of the aerobic C_4-dicarboxylate pathway encoding succinate dehydrogenase (*sdhCDAB*) and the aerobic succinate carrier (*dctA*) are only marginally or negatively regulated by the DcuSR system. The CitAB two-component regulatory system, which is highly similar to DcuSR, had no effect on C_4-dicarboxylate regulation of any of the genes.

In *Escherichia coli* the switch from aerobic to anaerobic metabolism is regulated at the transcriptional level in response to the presence of the electron acceptors O_2, nitrate, and fumarate (8, 9, 11, 25, 27, 28). This regulation ensures that in the presence of oxygen only aerobic metabolism and not anaerobic respiration or fermentation is functional. Under anoxic conditions, nitrate (and nitrite) represses the synthesis of the enzymes associated with fumarate respiration. The sensor-regulator systems controlling gene expression in response to O_2 and nitrate are known and have been studied in detail. Regulation by O_2 is effected by the two-component regulatory system ArcB/A (aerobic respiratory control) and by the cytoplasmic one-component regulator FNR (fumarate-nitrate reductase regulator) (8, 11, 27). Nitrate and nitrite regulate via two homologous two-component regulatory systems, NarX/L and NarP/Q (Nar is an acronym for nitrate reductase) (25).

Fumarate is also an important electron acceptor for respiration, and fumarate and related C_4-dicarboxylates are known to induce a variety of genes required for anaerobic fumarate metabolism, such as the structural genes for fumarate reductase (*frdABCD*) (8, 12), the proton-pumping NADH dehydrogenase I (*nuoA* to *-N*) (3, 26, 28), and dicarboxylate carriers (*dcu* genes) (7, 24, 29). In aerobic growth, synthesis of succinate dehydrogenase (*sdhCDAB*) is stimulated by the same substrates (18). Therefore, there is a large group of genes which should be transcriptionally regulated by fumarate or other C_4-dicarboxylates. For *Rhizobium leguminosarum* and *Rhodobacter capsulatus*, the two-component sensor-regulators, DctSR and DctBD, which control gene expression in response to C_4-dicarboxylates are known (10, 21). In the present study a two-component regulatory system was identified in *E. coli*. It is responsible for regulation of the genes of fumarate respiration, including fumarate reductase and a fumarate carrier (DcuB), in response to the presence of C_4-dicarboxylates.

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MATERIALS AND METHODS

Bacterial strains and growth. For genetic experiments the bacteria (Table 1) were grown aerobically in Luria Bertani broth (22). For expression studies the bacteria were grown in M9 mineral medium (15) supplemented with acid-hydrolyzed casein (1 g/liter) (26). Anaerobic growth was performed in gastight stoppered tubes under an atmosphere of N_2 (3). Aerobic growth was performed in flasks filled to 5% of the maximal volume with vigorous shaking. For anaerobic growth the carbon sources were added at 20 mM, and for aerobic growth the carbon sources were added at 10 mM. Cell densities were measured as the absorbance at 578 nm. Cells were harvested at an A_{578} of 0.5 to 0.7. β-Galactosidase assays were performed according to Miller (15).

Inactivation of *dcuR* (*yjdG*), *dcuS* (*yjdH*), and *citB*. The genes were inactivated by replacing their central portions with resistance cassettes. The flanking regions upstream and downstream of the genes were amplified by PCR. The downstream region of *dcuR* was amplified with primers yjdG-Hin (5′-TGA CAT CAA GAC CGC CCG AAG CTT GCA AGG-3′) and yjdG-Eco (5′-GCG TCC AGT TTA CCG TTA CCG AAT TCA GGC-3′), generating a 848-bp fragment with flanking *Hin*dIII and *Eco*RI sites. The upstream region of *dcuR* was amplified with primers yjdG-Pst (5′-TGT TCG TTG GAG CTG CAG CCG TGG ATT AGC-3′) and yjdH-Xba (5′-CAG TGA AAG CCA GCT TCT AGA CAG CGG CAG-3′), producing a 815-bp fragment with flanking *Pst*I and *Xba*I sites. The flanking region upstream of *dcuS* was amplified with primer YjdH-Eco (5′-CTC TCT GCG AAT TCT TTG TGC ATC-3′), introducing an *Eco*RI site, and primer YjdH-Bam-2 (5′-CTT CAG GAT CCG AGT AGC GAA GAC-3′), introducing a *Bam*HI site, generating a 1,091-bp fragment. The downstream flanking region of *dcuS* was amplified with primer YjdH-Xba (5′-TGA GCG CCT CTA GAA AGC GGG AAG-3′), with a *Xba*I site, and primer YjdH-Bam-1 (5′-GGC GTT ATC ATC GGA TCC ATT TC-3′), with another *Bam*HI site, generating a 1,020-bp fragment. The upstream region of *citB* was amplified with primers cri-Sac (5′-AAG ATG CTG GGG CTG AGC TCC-3′) and cri-Bam (5′-ATT CCG CAT GGA TCC CTG CC-3′), generating a 929-bp fragment with *Sac*I and *Bam*HI cloning sites. The downstream region of *citB* was amplified with primers cri-Hind (5′-ATG TTT AAA GCT TAT GCT CGC G-3′) and cri-Cla (5′ GAT CAT CGG TGT ATC GAT TTT TG-3′), producing a 918-bp fragment with *Hin*dIII and *Cla*I cloning sites. For each gene, the flanking regions were cloned into pKS$^-$ (Stratagene). For the *dcuR* and *citB* genes the Kan^r and Spc^r resistance cassettes derived from pGS607 and pGS606, respectively (24), were cloned into the *Eco*RI-*Pst*I and the *Bam*HI sites, respectively, resulting in *dcuR*::Kan^r (pMW75) and *citB*::Spc^r (pMW92). For *dcuS*, the flanking regions were separated by a single *Bam*HI site (pMW107). A Cam^r resistance cassette was amplified from pACYC184 (6) by PCR with primers CAMLIB2 (5′-CAA TAA CTG GAT CCA AAA AAT TAC GC-3′) and CAMREB (5′-ATA TCC TGG ATC CCA TAT TCT GC-3′), both introducing a *Bam*HI site. The resistance cassette was then cloned into the *Bam*HI site of pMW107, resulting in pMW108 (*dcuS*::Cam^r). Any possible terminating sequences downstream of the Cam^r resistance cassette were removed to enable transcription of *dcuR* located downstream of *dcuS*. The plasmids were transformed into *E. coli* JC7623 and were used for replacement of the intact genes by homologous recombination (16).

TABLE 1. Strains of *E. coli* and plasmids used

Bacterial strain or plasmid	Genotype	Reference or source
E. coli K-12		
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB530 deoC1 ptsF25 rbsR*	23
JC7623	*recB21 recC22 sbcB15 leu his thr pro arg ara*	16
IMW205	MC4100 but *dcuR*::Kan^r	This study
IMW262	MC4100 but *dcuS*::Cam^r	This study
IMW220	MC4100 but *citB*::Spc^r	This study
MC4100λJ100	MC4100 λ [Φ(*frdA′-′lacZ*)]	12
IMW206	MC4100 λ [Φ(*frdA′-′lacZ*)] but *dcuR*::Kan^r	IMW205(P1) × MC4100λJ100
IMW216	MC4100 λ [Φ(*frdA′-′lacZ*)] but *citB*::Spc^r	IMW220(P1) × MC4100λJ100
MC4100λPC33	MC4100 λ [Φ(*sdh*C′-′*lacZ*)]	18
IMW211	MC4100 λ [Φ(*sdh*C′-′*lacZ*)] but *dcuR*::Kan^r	IMW205(P1) × MC4100λPC33
IMW33	MC4100 λ [Φ(*nuo′-′lacZ*)]	3
IMW207	MC4100 λ [Φ(*nuo′-′lacZ*)] but *dcuR*::Kan^r	IMW205(P1) × IMW33
IMW237	MC4100 λ [Φ(*dcuB′-′lacZ*)]	This study
IMW238	MC4100 λ [Φ(*dcuB′-′lacZ*)] but *dcuR*::Kan^r	IMW205(P1) × IMW237
IMW239	MC4100 λ [Φ(*dcuB′-′lacZ*)] but *citB*::Spc^r	IMW220(P1) × IMW237
IMW240	MC4100 λ [Φ(*dcuC′-′lacZ*)]	This study
IMW241	MC4100 λ [Φ(*dcuC′-′lacZ*)] but *dcuR*::Kan^r	IMW205(P1) × IMW240
IMW260	MC4100 λ [Φ(*dcuB′-′lacZ*)] but *dcuS*::Cam^r	IMW262(P1) × IMW237
IMW261	MC4100 λ [Φ(*frdA′-′lacZ*)] but *dcuS*::Cam^r	IMW262(P1) × MC4100λJ100
Plasmids		
pJL28	*′lacZ*, protein fusion vector (Ap^r)	13
pJL29	*′lacZ*, protein fusion vector (Ap^r)	13
pMW75	pKS$^-$ but *dcuR*::Kan^r	This study
pMW92	pKS$^-$ but *citB*::Spc^r	This study
pMW108	pKS$^-$ but *dcuS*::Cam^r	This study
pMW99	pJL29 but *dcuB′-′lacZ*	This study
pMW98	pJL29 but *dcuC′-′lacZ*	This study
pMW103	pJL28 but *dctA′-′lacZ*	This study

Presence of the *dcuR*::Kan^r, *dcuS*::Cam^r, and *citB*::Spc^r alleles was confirmed by PCR of the genomic DNA with the corresponding primers, yielding fragments corresponding to the sizes of the inactivated genes. The inactivated genes were transferred to strains with suitable genetic backgrounds by P1 transduction (15).

Construction of protein fusions. For creating protein fusions (*dcuB′-′lacZ*, *dcuC′-′lacZ*, and *dctA′-′lacZ*) plasmid pJL28 or its derivative pJL29 was used (13). The *dcuB′-′lacZ* fusion was obtained by cloning the 0.65-kb PCR fragment generated with primer dcuB-Bam (5′-AAG TTG GAT CCT AAA TAA CAT GTG TGA ACC-3′) and primer yjdG-Eco into the *Bam*HI and *Eco*RI sites of pJL29, yielding pMW99. For the *dcuC′-′lacZ* fusion (pMW98), the *dcuB* promoter region was amplified with primers dcuC-Bam (5′-CCC CAA TAA GGA TCC CAA TG-3′) and dcuC-Eco (5′-CCA GCG GTG AAT TCC AGA CC-3′), and the 1.1-kb fragment was cloned into the *Bam*HI and *Eco*RI sites of pJL29. The *dctA′-′lacZ* fusion (pMW103) was obtained by cloning the 0.5-kb PCR fragment generated with primers dctA-Bam (5′-CAG AGA GGG ATC CAT AGG GTG TCC-3′) and dctA-Eco (5′-CGC TGG ATG AAT TCG GCA TGG G-3′) into the respective restriction sites of pJL28. The *dcuB′*- and *dcuC′-′lacZ* fusions were transferred to the chromosome with phage λRZ5 (12, 17), and monolysogens were identified and used for further work (3).

RESULTS

Fumarate induction of *dcuB* and *frdA* depends on the *dcuSR* regulatory genes. In a search for potential fumarate-responsive regulators, the *E. coli* data base was screened for gene products similar to the sensor-regulators DctRS and DctBD of *R. capsulatus* and *Rhizobium leguminosarum*, which stimulate the synthesis of the C_4-dicarboxylate carriers in response to C_4-dicarboxylates (10, 21). Both systems showed only low levels of similarity to two-component regulators of *E. coli* (<28% sequence identity). The genes for two of these systems, *yjdHG* and *citAB*, were located next to genes involved in anaerobic fumarate metabolism (Fig. 1). The *yjdHG* genes are in the *dcuB fumB* to *lysU* intergenic region at 93.7 min on the *E. coli* map (2). The *dcuB fumB* genes encode the anaerobically expressed fumarate carrier (*dcuB*) and fumarase (*fumB*) (1, 24). The *citA citB* (formerly *criR*) genes on the other hand are positioned at 14.1 min on the *E. coli* map between genes encoding an alternative fumarate carrier (*dcuC*) and the *citC* to *citT* gene cluster for anaerobic citrate metabolism (2, 20, 29). The *citAB* genes encode proteins homologous to the citrate sensor-regulators from *Klebsiella pneumoniae* (4, 5, 20). Anaerobic citrate metabolism of *E. coli* is related to C_4-dicarboxylate metabolism due to the production and excretion of succinate (5, 14).

The genes for the putative response regulator *yjdG* and the sensor kinase *yjdH* were genetically inactivated by replacement with genes carrying resistance cassettes. The mutant strains were tested for fumarate regulation of the *dcuB* and *frdA* genes (Table 2). Expression of the genes was determined with *lacZ* fusions, and growth was performed under anaerobic conditions on glucose or glycerol plus dimethyl sulfoxide (DMSO).

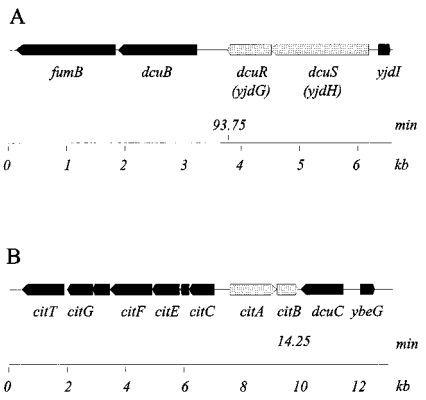

FIG. 1. Map positions and arrangement of the *dcuSR* (previously *yjdHG*) (A) and *citAB* (B) genes on the *E. coli* genome. The scale gives the DNA length in kilobases. The positions of the *dcuSR* and the *citAB* genes on the calculated *E. coli* map are shown. Data are from reference 2 and the *E. coli* data bank.

TABLE 2. Regulation of *dcuB* and *frdA* expression by fumarate, *dcuR* (formerly *yjdG*), and *dcuS* (formerly *yjdH*) under anaerobic conditions[a]

Substrate(s)	Regulation of *dcuB'-'lacZ* expression by:			Regulation of *frdA'-'lacZ* expression by:		
	WT[b]	*dcuR*	*dcuS*	WT	*dcuR*	*dcuS*
Glucose	8	5	2	150	130	130
Glucose + fumarate	45	3	1	300	155	150
Glycerol + DMSO	48	6	4	690	580	365
Glycerol + DMSO + fumarate	520	25	9	ND[c]	ND	ND
Glycerol + fumarate	560	7	18	1,010	400	430

[a] Expression of *dcuB* and *frdA* is given in Miller units. Growth occurred in M9 medium with the indicated substrates.
[b] WT, wild type.
[c] ND, not determined.

TABLE 3. Effects of C_4-dicarboxylates and DcuR on the expression of C_4-dicarboxylate-regulated genes

Gene fusion[a]	Substrate(s)[c]	β-Galactosidase activity (Miller units)	
		WT[d] (*dcuR*$^+$)	*dcuR*
nuoAB'-'lacZ	Glucose	95	96
	Glucose + fumarate	130	125
	Glycerol + fumarate	230	250
dcuC'-'lacZ	Glucose	52	52
	Glucose + fumarate	100	100
sdhC'-'lacZ	Glycerol + O_2	3,580	3,680
	Succinate + O_2	4,780	4,740
dctA'-'lacZ[b]	Glycerol + O_2	260	89
	Succinate + O_2	730	286

[a] MC4100 derivatives with *dcuR*$^+$ and *dcuR* backgrounds given in Table 1.
[b] Strains MC4100pMW103 and IMW205pMW103, respectively.
[c] M9 medium was used with the indicated substrates.
[d] WT, wild type.

DMSO has to be included as an electron acceptor for growth on glycerol, which cannot be fermented by *E. coli*. In the wild type, the expression of *dcuB* was stimulated 5.6-fold or 10.9-fold after growth on glucose or glycerol, respectively, when fumarate was present in the medium. When DMSO was omitted from the glycerol medium, a similar stimulation was found with the addition of fumarate. The lower expression of *dcuB* during growth on glucose could be due to glucose repression. In the *yjdG* (*dcuR*) and *yjdH* (*dcuS*) mutants the expression of *dcuB* was decreased to background levels, and the expression was not stimulated by fumarate (Table 2). Therefore both genes are required for fumarate stimulation of *dcuB* expression.

Expression of *frdA* is stimulated by the presence of fumarate in the medium, too, but this stimulation is lower (about twofold [Table 2]). In the *yjdG* (*dcuR*) and *yjdH* (*dcuS*) mutants background expression of *frdA* was still high, but the fumarate-dependent stimulation was lost completely.

The *citB* gene encoding the response regulator of the second two-component system (CitAB) was inactivated, too. The inactivation of *citB*, however, had no effect on expression and fumarate stimulation of the *dcuB* and *frdA* genes (not shown). Therefore, the *yjdHG* genes, but not the *citB* gene, are required for fumarate stimulation of *dcuB* and *frdA* expression. For this reason the genes *yjdH* and *yjdG* were termed *dcuS* (sensor kinase) and *dcuR* (response regulator).

Genes regulated by DcuR: not all C_4-dicarboxylate-regulated genes respond to DcuSR. Other genes which are transcriptionally regulated by C_4-dicarboxylates were tested in the same way for *dcuR* involvement (Table 3). The genes tested encode the proton-pumping NADH dehydrogenase I (*nuoA* to *-N* genes), an alternative anaerobic C_4-dicarboxylate carrier (*dcuC* gene), succinate dehydrogenase (*sdhCDAB*), and a C_4-dicarboxylate carrier for aerobic growth (*dctA*). The increase in the expression of the genes stimulated by fumarate or succinate was between 1.4- (*nuoAB'-'lacZ*) to 2.8-fold (*dctA'-'lacZ*) (Table 3). However, the fumarate- or succinate-dependent stimulation of *nuoA*, *dcuC*, and *sdhC* expression was not significantly affected in the *dcuR* mutant. Expression of the *dctA'-'lacZ* fusion was decreased in the *dcuR* mutant, but the succinate stimulation was retained and the increases were similar for the wild type (2.8-fold) and the mutant (3.2-fold). Thus, from the genes tested, only *dcuB* and *frdA* were clearly regulated by DcuR and DcuS. In the *citB* mutant neither of the genes was affected in C_4-dicarboxylate-stimulated expression (not shown).

C_4-dicarboxylates affecting regulation by DcuR. The effects of various carboxylates on the expression of *dcuB'-'lacZ* were studied by including the respective substrates in the medium (Table 4). Growth was performed under anaerobic conditions in the presence of glycerol plus DMSO, which enables high expression of *dcuB* when suitable carboxylates are added (see Tables 2 and 4). Each of the C_4-dicarboxylates fumarate, succinate, malate, tartrate, aspartate, and maleate caused a strong induction of *dcuB* expression compared to growth with glycerol plus DMSO alone. Even with succinate and maleate, which are not metabolized under the respective (anaerobic) conditions, the induction amounted to at least 63% of the maximal induction found with fumarate. Most remarkably, maleate, which is not even taken up by the anaerobic Dcu carriers (24), induced the expression of *dcuB* strongly. For all the C_4-dicarboxylates the stimulation was completely lost in the *dcuR* mutant. Therefore neither uptake nor metabolism of the C_4-dicarboxylates is required for induction by the DcuSR system. The results suggest that the C_4-dicarboxylates bind to the sensor at the periplasmic aspect of the membrane and that the sensor is able to react with each of the C_4-dicarboxylates. Butyrate and acetate, on the other hand, had no stimulating effect. During anaerobic growth on glucose, the respective C_4-dicarboxylates and aspartate showed similar stimulating effects, but expression was generally lower, possibly due to glucose repression (not shown). Expression of *frdA'-'lacZ* responded in a similar way to the C_4-dicarboxylates (not shown).

DISCUSSION

Physiology and significance of fumarate regulation in *E. coli*. Transcriptional regulation by fumarate and other C_4-dicarbox-

TABLE 4. Effectors for *dcuR*-dependent regulation of *dcuB'-'lacZ* expression during anaerobic growth with glycerol plus DMSO

Carboxylic acid in medium[a]	*dcuB'-'lacZ* expression (Miller units)	
	IMW237 (*dcuR*$^+$)	IMW238 (*dcuR*)
None	48	6
Fumarate	537	7
Succinate	437	4
Malate	435	4
Tartrate	382	13
Aspartate	434	10
Maleate	337	19
Butyrate	18	5
Acetate	26	3

[a] M9 medium was used as the growth medium.

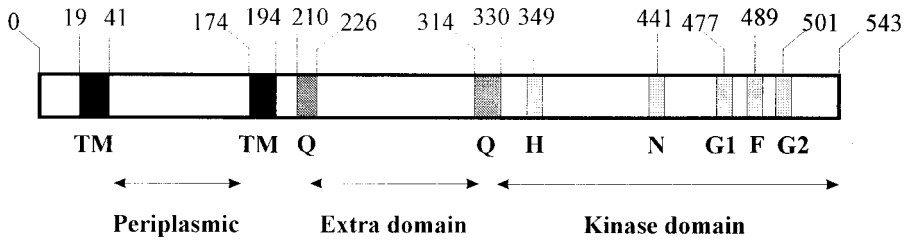

FIG. 2. Overview of the suggested domain structure of the sensor kinase DcuS. The positions of characteristic sequence features and of the domains are indicated by the numbers. The transmembrane helices (TM) were predicted from the sequence. The Q linkers (Q) and the signature segments of the kinase domain (H, N, G1, F, and G2) (19) were identified by sequence alignments. For the segments, the positions of the naming amino acid residue are given (drawn according to reference 5).

ylates plays an important role in *E. coli*. The DcuSR two-component regulators identified here apparently exert this fumarate regulation for the genes of fumarate respiration, that is, *frdABCD* and *dcuB*. The expression of *fumB* (encoding anaerobic fumarase B), which is located downstream of *dcuB* and is possibly expressed from the *dcuB* promoter (1, 24), could also be subject to DcuR regulation. Expression of other genes which are transcriptionally stimulated by C_4-dicarboxylates was not (*nuoA* to *-N*, *sdhCDAB*, and *dcuC*) or was only partially (*dctA*) dependent on DcuR. This indicates that DcuSR is required specifically for the regulation of the anaerobic fumarate respiratory pathway. The C_4-dicarboxylate regulation of other genes apparently is effected by a different system, and the CitA-CitB two-component regulatory system obviously does not serve this function either, as shown here.

DcuRS as a C_4-dicarboxylate-sensing two-component system. The *dcuSR* genes, and the derived DcuS and DcuR proteins, show the typical properties of two-component regulatory systems. Both genes overlap by 4 bp, which is a strong indication for a joint transcription similar to that of the genes of other two-component regulators, which are mostly organized in one transcriptional unit. The DcuR protein contains a helix-turn-helix DNA-binding motif in the C-terminal half and an N-terminal receiver domain with a conserved aspartate residue (Asp56) as a potential phosphorylation site.

The DcuS protein contains the elements typical for sensory histidine kinases, and the arrangement is very similar to that found in the CitA protein of *K. pneumoniae* (4, 5) (Fig. 2). The CitA protein consists of an N-terminal sensory domain with two transmembrane helices which are separated by a long periplasmic domain of about 130 amino acids (5). The kinase domain is separated from the sensor domain by an extra domain of about 80 amino acids. The similarity of DcuS to CitA extends over the complete range, including the periplasmic and the extra domain. In the kinase domain the H, N, F, and G boxes, which are designated according to the characteristic amino acid residues (19), are present in an arrangement very similar to that of CitA. His349, which is supposed to be the phosphorylation site, is conserved in the H box.

DcuS has significantly higher levels of similarity with the CitA citrate sensors of *K. pneumoniae* and *E. coli* than with the C_4-dicarboxylate sensors DctB and DctS of *Rhizobium* sp. strains and *R. capsulatus* (not shown). Both the citrate (CitA) and the C_4-dicarboxylate (DcuS, DctB, and DctS) sensors have similar N-terminal sensory domains consisting of two transmembrane helices and a long intervening periplasmic domain. The periplasmic domains of DctB and DctS, however, are about twice the size of the CitA or DcuS periplasmic domain, which presumably acts in ligand binding (5). Such a location of the sensory domain in the periplasm suggests sensing of the C_4-dicarboxylates from without. This is also supported from the functioning of maleate as a signal which apparently is not taken up by the bacteria (24). In agreement with the postulated fumarate sensing by DcuSR from without, the fumarate carrier (DcuB) shows a strong induction by fumarate (up to 10.9-fold), whereas fumarate reductase shows only a weak induction by fumarate (up to twofold). These different responses to external fumarate appear to be sensible since DcuB is required in particular when external fumarate is present. FrdA on the other hand is also required when internal fumarate is produced from intermediary metabolism.

ACKNOWLEDGMENTS

This study was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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